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Peroxidase 4 is involved in syringyl lignin formation in *Arabidopsis thaliana*

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ABSTRACT

Syringyl lignins result from the oxidative polymerization of sinapyl alcohol in a reaction mediated by syringyl (basic) peroxidases. Several peroxidases have been identified in the genome of *Arabidopsis thaliana* as close homologues to ZePrx, the best characterized basic peroxidase so far, but none of these has been directly involved in lignification. We have used a knock-out mutant of AtPrx4, the closest homologue to ZePrx, to study the involvement of this basic peroxidase in the physiology of the plant under both long- and short-day light conditions. Our results suggest that AtPrx4 is involved in cell wall lignification, especially in syringyl monomer formation. The disruption of *AtPrx4* causes a decrease in syringyl units proportion, but only when light conditions are optimal. Moreover, the effect of *AtPrx4* disruption is age-dependent, and it is only significant when the elongation process of the stem has ceased and lignification becomes active. In conclusion, AtPrx4 emerges as a basic peroxidase regulated by day length with an important role in lignification.

Introduction

Lignins are three-dimensional phenolic heteropolymers resulting from the oxidative coupling of three *p*-hydroxycinnamyl (*p*-coumaryl, coniferyl and sinapyl) alcohols (*i.e.* monolignols). The cross-coupling reaction of monolignol radicals produces a hydrophobic heteropolymer composed of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units (Vanholme et al., 2010).

Lignins are covalently associated with polysaccharides in plant cell walls, they impart water impermeability, including resistance against tensile forces of the water columns and confer structural support and flexural stiffness to the aerial organs (Boerjan et al., 2003). These polyphenolic compounds are deposited mainly in tracheids, vessels, fibres of the xylem and phloem and schlerenchyma (Boerjan et al., 2003). However, lignin composition varies among species, phylogenetic groups, cell types, development stage and even seasonal growth. Different stresses also provoke a change in lignin composition, depending on the stress type (Moura et al., 2010).

Even though the whole route has already been dissected, new data confirm the complexity of the biosynthetic pathway, not only due to the appearance of new enzymes in non-angiosperm species (Weng et al., 2010), but also for new catalytic activities of different enzymes (Zhou et al., 2010) and the description of novel enzymes that participate in monolignol formation (Vanholme et al., 2013). In fact, the recent description of caffeoyl shikimate esterase (CSE) as a crucial enzyme in the lignin biosynthetic pathway forces researchers to revise the currently accepted models of this biosynthetic pathway (Vanholme et al., 2013).

The last step of lignin biosynthesis is the oxidation of monolignols, which is driven by laccases (Berthet et al., 2012) and peroxidases (Fagerstedt et al., 2010). However, while peroxidases are able to oxidize monolignols to produce H, G and S units, laccases only generate G units (Berthet et al., 2011). It is worth mentioning that not every peroxidase isoenzyme has the ability to oxidize the three monolignols. While the whole spectrum of peroxidases, anionic and cationic, is able to oxidize coniferyl and *p*-coumaryl alcohols *in vitro*, only few peroxidases are able to use sinapyl alcohol as substrate. Most peroxidases have been described as guaiacyl peroxidases, which oxidize preferentially

Abbreviations: 4CL, 4-hydroxycinnamoyl-CoA ligase; AtPrx, *Arabidopsis thaliana* peroxidase; CAD, cinnamyl alcohol dehydrogenase; COMT, caffeic acid/5-hydroxyferulic acid O-methyltransferase; F5H, ferulic acid 5-hydroxylase; FTIR, Fourier transform infrared spectra; G, guaiacyl; H, hydroxyphenyl; PAL, phenylalanine ammonia lyase; S, syringyl; WT, wild type; ZePrx, peroxidase from *Z. elegans*.

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coniferyl over sinapyl alcohol. Peroxidases that catalyze the formation of syringyl moieties by the oxidation of sinapyl alcohol are called syringyl peroxidases. The latter have been found widespread in plant evolution, but have been isolated only in few species, such as *Betula pendula* (BPX1) (Marjamaa et al., 2006), *Populus alba* (CWPO-C) (Sasaki et al., 2004) and *Zinnia elegans* (ZePrx) (Gabaldón et al., 2005). These three peroxidases oxidize sinapyl alcohol as their preferred substrate and there have been linked to lignification of cell walls. However, the best characterized is ZePrx, which shows all the features to be considered responsible for the biosynthesis of lignin in the xylem cell walls (H_2O_2-dependent oxidase activity, ability to oxidize sinapyl alcohol, low K_M values and structural determinants in the amino acid sequence) (Gabaldón et al., 2005; Gómez-Ros et al., 2007). Since such a peroxidase similar to ZePrx has not been yet identified in *Arabidopsis*, Herrero et al. (2013a) searched through *A. thaliana* genome for peroxidases showing the highest homology to ZePrx. Based on several structural and molecular characteristics, peroxidase 4 (AtPrx4 according to PeroxiBase nomenclature) was determined to be the most homologous to ZePrx in *Arabidopsis thaliana* (Herrero et al., 2013a).

On the other hand, light plays an important role in different plant processes, including the phenylpropanoid metabolism that leads to lignin biosynthesis (Hemm et al., 2004). Light increases the proportion of tracheary elements differentiating in *Pinus radiata* xylem-derived callus (Möller et al., 2006), as well as induces the enzymatic activity and gene expression of phenylalanine ammonia lyase (PAL), 4-hydroxycinnamoyl-CoA ligase (4CL) and cinnamyl alcohol dehydrogenase (CAD), all of them key participants in lignin biosynthesis (Möller et al., 2006; Hemm et al., 2004).

Moreover, lignins also affect the use of plant biomass as a source for biofuels and hinder the digestibility of animal feed. Therefore, many attempts have been made in the last years to alter lignification and make cell wall more suitable to facilitate its use as biomass (Li et al., 2008). The identification of genes responsible for lignin biosynthesis is therefore essential to allow metabolic engineering of lignins.

Here, we study the function that AtPrx4 may have in plant physiology by using an *Arabidopsis* knock-out mutant, and how plants in absence of *AtPrx4* expression lignify in both long- and short-day conditions.

Materials and methods

Plant material

Seeds of *A. thaliana* Columbia 0 (Col-0) ecotype and *atprx4* mutant (Salk_044730.56.00) (Alonso et al., 2003) were obtained from the Nottingham Arabidopsis Stock Centre (NASC) on-line catalogue at http://nasc.nott.ac.uk/home.html. Seeds were sterilized, washed and kept in cold conditions (4 °C) for 3 days (Harrison et al., 2006) before culture. Plants were grown under controlled conditions with a day/night temperature regime of 22/20 °C, 70% HR and under a long- or short-day photoperiod of white light (110 μmol m^{-2} s^{-1}) in a Conviron CMP6050 growth chamber.

In order to select homozygous mutant plants, *atprx4* seeds were placed on solid MS medium supplemented with 0.1% (w/v) sucrose and with 50 μg mL^{-1} of kanamycin (Sigma–Aldrich, Madrid, Spain) as selection agent and exposed to 6 h light to induce germination, and then maintained for 2 days in darkness at 24 °C as described by Harrison et al. (2006). One week after germination, plants were transferred to soil and maintained until needed for the experiments.

RNA isolation and cDNA synthesis

Total RNA was isolated from different organs with Trizol (Invitrogen) as described by Chomczynski (1993) with minor modifications. RNA integrity was checked by electrophoresis in 1.5% agarose gel and quality was confirmed with a Bioanalyzer instrument (Bioanalyzer 2100 of Agilent Technologies). cDNA was synthesized from 1 μg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas).

Gene expression analysis by q-PCR

The cDNA samples were analyzed with the 7500 Fast Real Time PCR System (Applied Biosystems) using SYBR Green PCR Core Reagents (Life Technologies). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C. For each mRNA, gene expression was normalized to actin in each sample. The data analysis was carried out with the Applied 7500 System SDS 2.0.5. The primer efficiency was calculated and the resulting Ct values were processed according to Pfaffl (2001) to obtain the relative expression values. The primers used are shown in Table S1 in Supplementary Information. In all cases, each PCR was performed with triplicate samples and repeated with at least three independent samples.

Histochemical assays for detecting lignins

Lignins were detected in inflorescence stem sections using the toluidine blue, Wiesner and Mäule stainings. Three different biological experiments were performed, five wild type (WT) and five *atprx4* plants were selected and at least 20 sections from each plant were observed. Cross sections were taken from the 5 cm basal part of stems collected at development stage 6.3 (Boyes et al., 2001), corresponding to a stem height of 15 cm. Briefly, stems were fixed in McDowell solution during 24 h and post-fixed with 1% OsO_4 in the same conditions. Following post-fixation, samples were stained with uranyl acetate, dehydrated and embedded in spurr as described by Herrero et al. (2013b). Thin sections (5 μm) were examined, after staining with a toluidine blue solution, with a Leica DMRB optical microscopy.

Wiesner staining was performed by soaking 60 μm thick sections in 1.0% (w/v) phloroglucinol–HCl in 25:75 (v/v) HCl/ethanol for 10–15 min (Pomar et al., 2002). Lignins were also stained with the Mäule test, a specific reaction for syringyl moieties. Sections were incubated in 1% $KMnO_4$, washed with 30% HCl and observed in ammonia (Pomar et al., 2002).

Electron microscopy

In order to study stem cell wall structure by electron microscopy, stems were prepared as described above for toluidine blue staining. Following post-fixation, samples were stained with uranyl acetate and lead citrate for 15 min. Ultrathin sections (500 nm) were examined with a Philips Tecnai 12 electron microscope operating at 60 kV.

Phenotypic analysis

Development analyses of at least 50 WT and *atprx4* plants were carried out as described by Boyes et al. (2001). Determination of rosette area was performed with Image J 1.47 software.

Lignin analyses

Cell walls were prepared with a Triton X-100 washing procedure in which the final steps include washing with ethanol (three times)

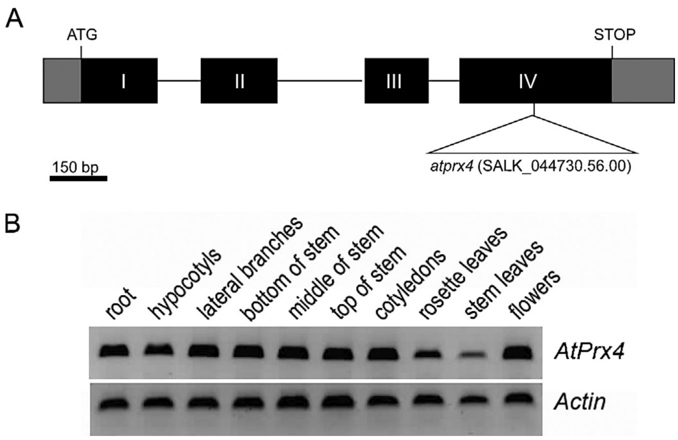

Fig. 1. (A) Description of *atprx4* T-DNA insertion mutants. Position of the T-DNA insertion site in *atprx4*. Grey boxes, 5′ and 3′ untranslated regions; black boxes, exons; lines, introns. (B) Localization of *AtPrx4* transcripts in different organs of WT plants.

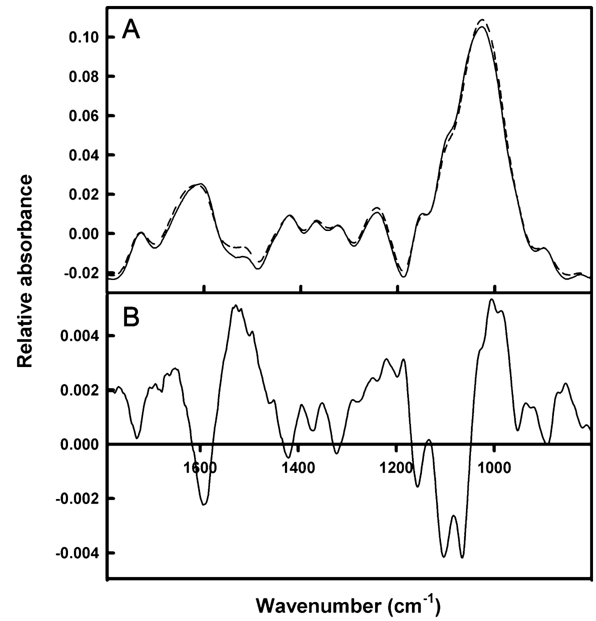

Fig. 2. Fourier transform infrared spectra of cell walls of WT (continuous line) and *atprx4* (broken line) plants (A) and the difference spectrum (B). The difference spectrum was generated by subtracting the mean spectrum of cell walls of *atprx4* plants from that WT plants. Each spectrum is a mean of spectra from five individual samples.

and diethyl ether (also three times) (Herrero et al., 2013b). Lignin quantification was performed using acetyl bromide, as described by Iiyama and Wallis (1988). Thioacidolysis of lignifying cell walls, which solubilizes the ß-*O*-4 lignin core, and GC-MS analyses were performed (Novo-Uzal et al., 2009) using a Thermo Finnigan Trace GC gas chromatograph, a Thermo Finnigan Polaris Q mass spectrometer, and a DB-XLB, J&W (60 m × 0.25 mm I.D.) column. Mass spectra were recorded at 70 eV. Fourier transform infrared spectra (FTIR) of finely ground cell wall samples were recorded on a Bruker Vector 22 FTIR spectrophotometer (Bruker Optics, Madrid, Spain).

Accession numbers

The Arabidopsis Genome Initiative locus identifiers or the GenBank database accession numbers for the genes investigated in this study are PRX4 (At1g14540), PAL1 (At2g37040), 4CL1 (At1g51680), CCR1 (At1g15950), F5H1 (At4g36220), CAD6 (At4g34230), CesA7 (At5g17420), CesA8 (At4g18780), FRA8 (At2g28110), CHS (At5g13930), SMT (At2g22990), MYB58 (At1g16490), MYB103 (At1g63910), SND1 (At1g32770) and Actin (At1g49240).

Statistical analysis

Experiments were repeated at least three times, selecting at least 50 plants. All data are given as the mean ± SD. Data were analyzed by Student's *t* test to confirm data variability and comparisons of means. All statistical analyses were performed with SPSS v.13.0.

Results

AtPrx4 is homologous to ZePrx and expressed in stems

The gene encoding for AtPrx4 is localized in chromosome 1 and has the classical structure of four exons and three introns (Fig. 1A). It has been reported to show close homology to ZePrx (Herrero et al., 2013a), which is highly expressed in hypocotyls and epicotyls. However, no bibliographic data was available concerning *AtPrx4* expression in *Arabidopsis* stems, although it has been reported to be expressed in roots (Welinder et al., 2002; Armengaud et al., 2004; Yang et al., 2011), leaves (Ramírez et al., 2011; Rasul et al., 2012; Wang et al., 2012) and cell cultures (Guan and Nothnagel, 2004). Moreover, this protein is induced in normal development but also under several stress conditions, such as hypoxia, oxidative stress and pathogen infection (Ramírez et al., 2011; Yang et al., 2011; Rasul et al., 2012; Li et al., 2013). In order to determine the localization of *AtPrx4* transcripts, RNA was extracted from different organs of WT plants. Our results showed that *AtPrx4* expression was localized in roots, hypocotyls, lateral branches, inflorescence stem, cotyledons, leaves and flowers (Fig. 1B).

Disruption of AtPrx4 does not affect plant growth but causes a reduction in lignin content

A line with a T-DNA insertion in the exon 4 of *AtPrx4* (Fig. 1A) was chosen to proceed with the functional characterization of the mutant plant. In plants grown under long-day photoperiod, the rosette growth was completed 31 days after sowing, in agreement with Boyes et al. (2001), corresponding to a development stage 3.9 (Supplementary Information Fig. S1A). The inflorescence stem emerged 38 days after sowing and its growth was completed 56 days after sowing (Supplementary Information Fig. S1B). WT and *atprx4* plants were indistinguishable in appearance, showing no differences regarding area and major axis of the rosette, as well as stem length and number of lateral branches (Supplementary Information Fig. S1). Plants grown under short-day conditions showed a similar pattern (data not shown).

We performed an analysis of the composition of isolated cell walls by means of FTIR spectra in the finger print range from 1800 to 800 cm^{-1} (Fig. 2A). Some differences occurred in the carbohydrate regions between 800 and 1200 cm^{-1} (Fig. 2B). Within this region, cellulose, pectin, rhamnogalacturonan and xyloglucan have overlapping peaks (McCann et al., 1992) and therefore, the observed alterations cannot be assigned to distinct compounds. Moreover, some alterations were found in the 1400–1650 cm^{-1}, which corresponds to vibration in the aromatic skeleton in lignin and a C═O stretch attributable conjugated/aromatic aldehydes, where the carbonyl oxygen atom sustains either intramolecular or intermolecular H-bonds, as well as in-plane bending in the C—H group (Rana et al., 2010). All these results suggest WT cell walls are richer in carbohydrates and lignin than *atprx4* cell walls.

Table 1
Lignin content, determined by the acetyl bromide method and expressed in μg lignin mg^{-1} cell wall, of WT and *atprx4* plants grown under short- or long-day light conditions. Data presented are average values ± SD of $n = 3$ experiments. Asterisk indicates significant differences at $P < 0.05$ determined by the Student's *t* test.

	Short-day	Long-day
WT	134 ± 19	145 ± 14
atprx4	134 ± 14	91 ± 11*

It is well known that light induces both the phenylpropanoid metabolism and the specific pathway of lignin biosynthesis (Hemm et al., 2004). For this reason, by means of acetyl bromide method (Iiyama and Wallis, 1988), we also quantified the lignin content in isolated cell walls, from the inflorescence stem of both mutant and WT plants grown under long- and short-day light conditions (Table 1). According to literature and previous results, the amount of lignins in short-day WT plants was slightly lower than in long-day plants. The *atprx4* plants grown under short-day conditions showed no differences in comparison with WT plants (134 μg lignin mg^{-1} CW), whereas *atprx4* plants grown under long-day conditions decreased its lignin content (37%) in comparison with WT plants (Table 1).

However, the disruption of *AtPrx4* only seems to affect lignin content when plants are grown under long-day conditions, suggesting the importance of AtPrx4 in lignification but only in specific environmental conditions.

Disruption of AtPrx4 causes a reduction of syringyl unit proportion

A reduction in lignin content can be accompanied by a change in monomer composition, even more, sometimes no change in lignin content however involves a change in lignin monomer composition. Therefore, we have performed thioacidolysis to cell walls, which allows the study of both the composition and structure of the lignin polymer by solubilizing lignins by the cleavage of β-ether linkages (Lapierre et al., 1995).

No differences were found between *atprx4* and WT mature plants (about 8 weeks) grown under short-day conditions (Table 2). However, when plants were grown under long-day conditions, some changes were observed between *atprx4* and WT plants . In fact, an increase of G units proportion was accompanied by a decrease in S units proportion in *atprx4* plants. This was translated into a decrease in S/G ratio, from 0.65 in WT plants to 0.48 in *atprx4* plants.

Taking into account that the differences were only found when plants were grown under long-day photoperiod, we tested if these differences were time-dependent. In this way, plants with 15 cm long floral stems were divided into three sections of 5 cm each, from basal to apical parts. Cell walls were isolated and analyzed by means of thioacidolysis (Table 3). Results showed changes in monomer composition specifically in the 5 cm basal part of the floral stem, which mirrors the results of mature plants, with an increase of G units proportion accompanied by a decrease in S units proportion. S units are incorporated into the lignin polymer in the process so called "end-wise polymerization," where lignin polymerization takes place when the monomers are added constantly for a period of time, and the subsequent growing lignin polymer is linked by β-*O*-4 ether bonds with a low number of end groups. Usually, middle lamella and primary cell walls are constituted by lignin formed in a bulk polymerization, with many condensed structures (C—C) and mainly composed of G units (Sarkanen, 1971). On the contrary, syringyl-rich cell wall lignins are more linear, with higher number of β-*O*-4 bonds and formed by an end-wise polymerization. The basal part of the stem contains thicker secondary cell walls and the percentage of S units as well as the S/G ratio is higher in the WT. This could explain why the differences between *atprx4* and WT plants are restricted to basal parts. AtPrx4 is homologue to the syringyl peroxidase ZePrx, and has been reported to fulfil all the structural requirements to be a syringyl peroxidase itself. The reduction in S units proportion in floral stems in *atprx4* fits well with the suppression of *AtPrx4* expression, suggesting the involvement of AtPrx4 in lignification of cell walls, particularly in oxidation of sinapyl alcohol to render S monomers.

Table 2
Cell wall monomer composition, expressed as the molar H:G:S and S/G ratios determined by thioacidolysis, of WT and *atprx4* plants grown under short- or long-day light conditions. Asterisk indicates significant differences at $P < 0.05$ determined by the Student's *t* test.

	H:G:S		S/G ratio	
	Short-day	Long-day	Short-day	Long-day
WT	1:66:33	1:60:39	0.50	0.65
atprx4	1:65:34	2:66:32	0.52	0.48*

Disruption of AtPrx4 affects interfascicular fibres

Due to the fact that in angiosperms most syringyl lignins are located in the fibres, whereas xylem is mainly composed of guaiacyl lignin, histochemical analyses were performed to test which tissues were affected the most by the disruption in *AtPrx4*. Consistently with lignin quantification and thioacidolysis analyses, no differences were found between *atprx4* and WT plants grown under short-day conditions (data not shown). Regarding plants grown under long-day conditions, toluidine blue staining showed no differences between *atprx4* and WT cell walls. Phloroglucinol staining, which detects cinnamyl aldehyde end groups, revealed that the presence of lignins was restricted to vascular bundles and interfascicular fibres (Fig. 3C and D), with lower intensity in the interfascicular fibres of the knock-out mutant. Since AtPrx4 is a basic peroxidase which putatively oxidizes sinapyl alcohol, the Maüle test, which is specific for syringyl groups, was performed (Pomar et al., 2002). Xylem vessel were brown-stained, which points out that lignins are mainly composed of guaiacyl units, whereas red staining in the interfascicular fibres indicated the presence of syringyl groups, both in WT and *atprx4* plants (Fig. 3E and F). However, unlike what thioacidolysis pointed out, no remarkable differences were found between WT and *atprx4* plants. While interfascicular fibres are mainly constituted by S lignins, xylem is mainly composed of G units (Zhong et al., 2000), which would explain why xylem vessels were equally stained in both WT and mutant plants while interfascicular fibres show lower intensity when they are stained with phloroglucinol.

A detailed analysis of ultrastructure of xylem vessels and interfascicular fibres cell walls was performed with transmission electron microscopy. No clear differences were found between WT and *atprx4* plants, but some changes occurred (Fig. 4). In the *atprx4* interfascicular fibres, middle lamella was less electron-dense than in WT plants. Taking into account that middle lamella is the initiation point for lignification, this could indicate a delay in lignification pattern in interfascicular fibres (Fig. 4). Moreover, the border between primary and secondary cell wall is more diffuse in *atprx4* plants, suggesting some minor changes at the level of cellulose patterning. In fact, some changes in carbohydrate composition were revealed by FTIR spectra of isolated cell walls (Fig. 2).

AtPrx4 suppression causes a down-regulation of genes involved in lignin biosynthesis

In order to test if the suppression of the last enzyme of the route leads to a feedback repression of the whole route or to a reallocation of the intermediates, we analyzed the expression of some

Table 3
Cell wall monomer composition, expressed as the molar G:S and S/G ratios determined by thioacidolysis, of bottom, middle and top parts of 15 cm inflorescence stems of WT and *atprx4* plants grown under long-day light conditions. Data presented are average values ± SD of $n = 3$ experiments. Asterisk indicates significant differences at $P < 0.05$ determined by the Student's t test.

	G:S						S/G ratio		
	Bottom		Middle		Top		Bottom	Middle	Top
	G	S	G	S	G	S			
WT	70 ± 3	30 ± 3	81 ± 1	19 ± 1	93 ± 1	7 ± 1	0.43	0.23	0.08
atprx4	75 ± 1	25 ± 1	85 ± 4	15 ± 4	94 ± 0	6 ± 0	0.33*	0.18	0.06

genes involved in lignin biosynthesis, such as *PAL, 4CL, CCR, F5H* and *CAD*. Results showed (Fig. 5A) a down-regulation of all the genes involved in lignin biosynthesis, from the entry point of phenylpropanoid metabolism (*PAL*), until the specific genes involved in S monomers biosynthesis (*F5H*). The *F5H* down-regulation, along with the suppression of *AtPrx4* may explain the decrease in S units shown in Table 2.

The finding of carbohydrates alterations in cell wall prompted us to analyze the expression levels of secondary wall biosynthetic genes. Real-time quantitative PCR analysis demonstrated that the secondary wall biosynthetic genes, including cellulose synthases (*CesA7* and *CesA8*) and xylan biosynthetic genes (*FRA8*) were all down-regulated in *atprx4* plants compared with the wild type (Fig. 5B). This down-regulation is in agreement with results obtained with FTIR, in which WT showed higher carbohydrate peaks than *atprx4* plants (Fig. 2).

In order to decipher if some other routes of phenylpropanoid metabolism were up-regulated due to a down-regulation of lignin biosynthesis genes, expression levels of *CHS*, which is involved in flavonoid biosynthesis, and *SMT8*, involved in sinapate esters formation, were determined by qPCR. In *atprx4* plants, *SMT8* was slightly up-regulated, while CHS showed no differences in comparison with WT (Fig. 5B).

Moreover, gene expression of transcription factors was analyzed, showing the down-regulation of *MYB58* and *SND1* (Fig. 5B), which regulate the expression of lignin biosynthetic genes and

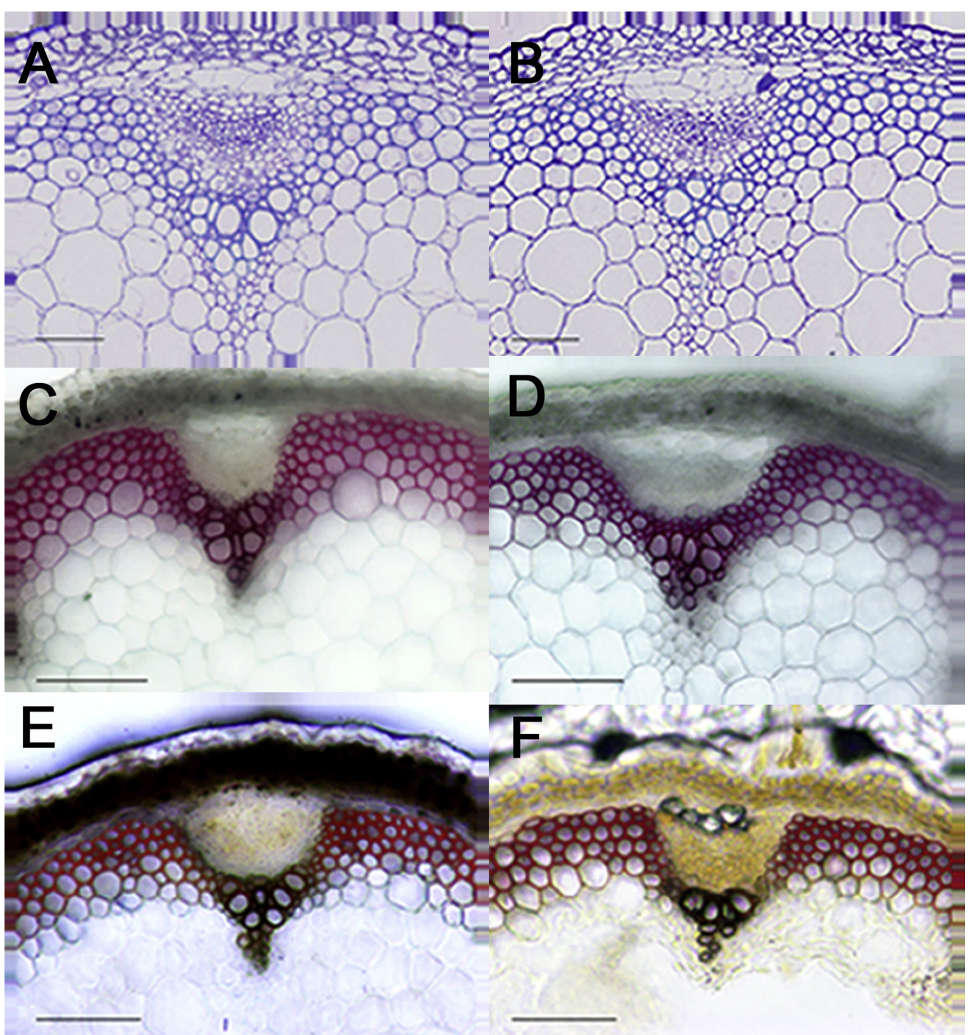

Fig. 3. Histochemical staining in WT (A, C, E) and *atprx4* (B, D, F) grown under long-day conditions, with toluidine blue (A, B), phloroglucinol-HCl (C, D) and Mäule stainings (E, F). Bars: 100 µm.

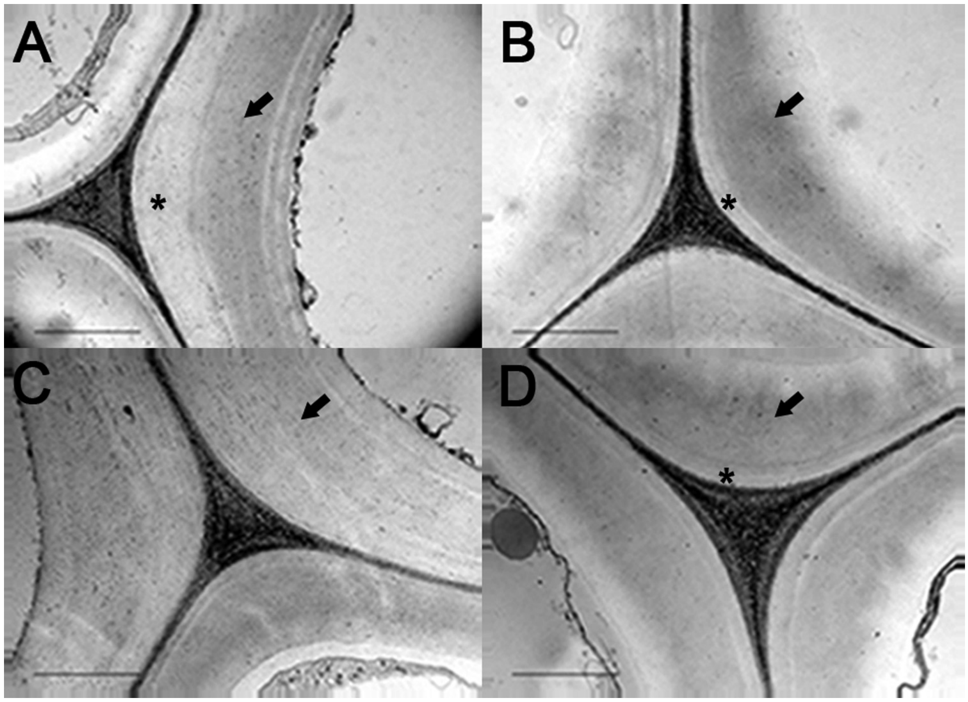

Fig. 4. Transmission electron microscopy of secondary walls of vessels (A, C) and interfascicular fibres (B, D) of WT (A, B) and *atprx4* (C, D). Asterisk shows primary cell wall and arrows show secondary cell wall in WT and mutant plants grown under long-day conditions. Bars: 1 μm.

secondary cell wall thickening in fibres, respectively (Zhong et al., 2006; Zhou et al., 2009).

Discussion

Lignins present in cell walls of *Arabidopsis* are mainly formed of coniferyl and sinapyl alcohol, with a minor contribution of *p*-coumaryl alcohol. Although coniferyl and sinapyl alcohol only differ in the presence of a second O methyl group in sinapyl alcohol, it affects chemical properties, monomer proportion and mechanism of synthesis. The presence and proportion of S monomers in lignins widely differ among taxonomic plant groups (Novo-Uzal et al., 2012), but also among different cell types and layers of the cell walls. The formation of S monomers requires the existence of specific enzymes, ferulic acid 5-hydroxylase (F5H) and caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT), which catalyze the formation of sinapaldehyde from coniferaldehyde. Sinapaldehyde is then reduced by CAD into sinapyl alcohol, which is subsequently oxidized by syringyl peroxidases. These are usually strongly basic proteins and show no steric restrictions that hinder the entry of sinapyl alcohol into the catalytic centre (Ros Barceló et al., 2007). Here, we report that the insertion of T-DNA in *AtPrx4*, which encodes a strongly basic peroxidase, causes a reduction of both lignin content and S/G ratio. Several attempts have been made by metabolic engineering to study the function of these basic peroxidases in lignification, with different outcomes. The antisense suppression of a basic peroxidase in tobacco caused a reduction of lignin levels (Talas-Ogras et al., 2001) and the major physiological change produced by the down-regulation of a basic peroxidase in tobacco was on overall lignin deposition, which decreased up to 50% together with a reduction of the levels of G and S units (Blee et al., 2003). Moreover, the down-regulation of the acidic peroxidase PrxA3a reduced up to 20% lignin content in transgenic lines of aspen, but only G units were decreased (Li et al., 2003). Here, we report that the decrease in lignin content was about 35%, which is similar to the decrease in lignin content in other peroxidase mutant lines. In fact, a similar reduction of lignin content in *Arabidopsis* plants was observed when the expression of another syringyl peroxidase, AtPrx72, was suppressed (Herrero et al., 2013b).

On the other hand, the age of the plant also seems to be important for detecting changes in lignin content. In fact, *Arabidopsis* plants with 6 weeks in which *CCR* was suppressed showed a reduction of lignin amount, although this difference was indistinguishable in 9-week plants (Laskar et al., 2006). These results suggested a delay in lignification pattern without affecting the plant capacity to overcome this gene expression decrease, since normal levels of lignins were reached at complete maturity. However, we have used mature plants (around 8 weeks), which correspond to development stage 6.9 according to Boyes et al. (2001), and differences in lignin content were still detected, pointing out the fundamental role of peroxidases in the oxidative coupling of monolignols.

Moreover, the differences in lignification were not only restricted to acetyl bromide lignin content, but also monomer composition, decreasing the percentage of S units and subsequently decreasing the S/G ratio. The lignin biosynthetic pathway is common for G and S units until the formation of coniferaldehyde, where the route is divided into two different pathways (Boerjan et al., 2003). Therefore, the major changes in monomer composition found in mutant plants occur when mutations affect the genes encoding for the last enzymes of the route, such as CAD, F5H, COMT and peroxidases. As F5H and COMT are specific for sinapyl alcohol formation, a mutation in any of these two genes leads to a severe diminution of S units and S/G ratio (Piquemal et al., 2002). CAD mutants usually incorporate aldehydes (coniferaldeyde and sinapaldehyde) into the lignin polymer, but display differences depending on the affinity of the enzyme for coniferaldehyde or sinapaldehyde. There are some CAD enzymes which prefer sinapaldehyde over coniferaldehyde as substrate (also named SAD), and *cad* lignins usually lack S units (Sibout et al., 2005; Fornalé et al., 2012). However, when CAD uses preferentially coniferaldehyde no change in monomer composition is observed in *cad* mutants (Marita et al., 2003).

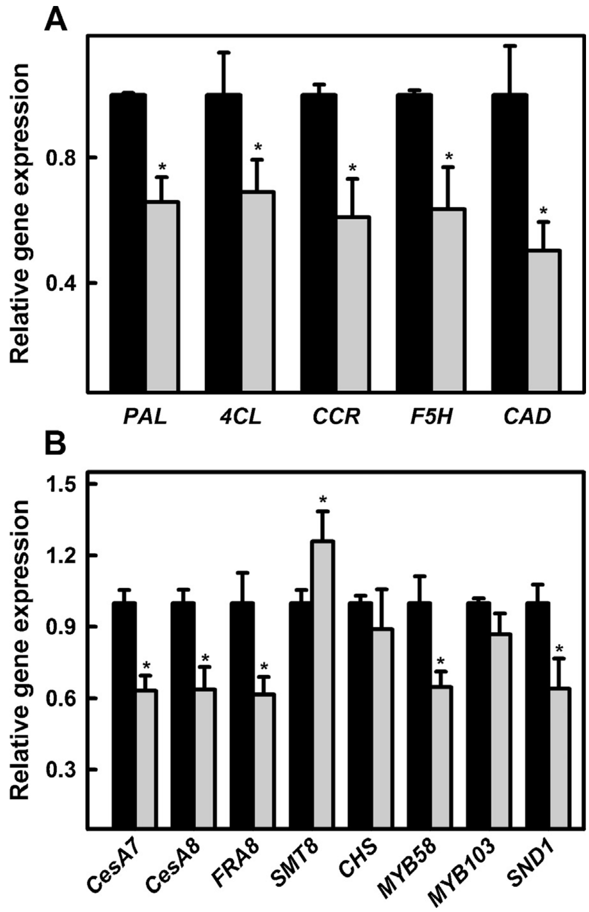

Fig. 5. Effect of *AtPrx4* suppression on the expression of genes involved in phenylpropanoid pathway, secondary wall biosynthesis and transcriptions factors. (A) Real-time quantitative PCR analysis of the expression of lignin biosynthesis genes. (B) Real-time quantitative PCR analysis of the expression levels of secondary wall biosynthesis genes, phenylpropanoid pathway and transcription factors. Actin was used as reference gene. Error bars represent SD of three replicates. Asterisks indicate significant differences at $P < 0.05$ determined by the Student's *t* test.

The suppression of basic peroxidases usually leads to a decrease in both lignin content and S/G ratio by decreasing S monomers (Herrero et al., 2013b; Blee et al., 2003), but suppression of acidic peroxidases provokes a change in lignin content, mainly due to a diminution of G units (Li et al., 2013). Here, we have demonstrated that the T-DNA insertion in *AtPrx4*, which encodes a basic peroxidase, causes a decrease in S/G ratio (Tables 2 and 3), suggesting the participation of AtPrx4 in sinapyl alcohol oxidation.

According to histochemical analysis, the main changes in monomer composition occurred in interfascicular fibres, as observed in the lower intensity of Wiesner staining (Fig. 3C and D). Interfascicular fibres are mainly composed of S monomers, therefore the lower intensity shown by Wiesner staining in *atprx4* plants agrees with the high homology that AtPrx4 shares with ZePrx, considering that ZePrx is a syringyl peroxidase that oxidizes sinapyl alcohol to render S units. Nonetheless, in xylem, which is composed of G units, no differences were observed (Fig. 3C and D). Similar results were found for knock-out *atprx72* mutants, showing that the mutation only affects the interfascicular fibres, decreasing S units, but xylem remained unaltered (Herrero et al., 2013b). It has been shown that AtPrx72 is one of the *Arabidopsis* peroxidases which shows strong homology to ZePrx (Herrero et al., 2013a).

The effect of *AtPrx4* mutation was age-dependent, as the only visible effects were restricted to the basal part of the inflorescence stems (Table 3). When internodes were still elongating (top and middle parts) and growth-related metabolism was occurring rather than lignin biosynthesis, *AtPrx4* mutation had no effect on plant physiology. However, when lignin biosynthesis becomes an active process and elongation has ceased (bottom part), differences are apparent. This expression pattern suggests the involvement of AtPrx4 in lignification of cell walls.

Even though there are differences in lignin content, no significant differences were found in the size and growth of WT and mutant plants (Supplementary Information Fig. S1). In this way, many plants which have alterations in lignin biosynthetic pathway show no difference with WT regarding diverse growth parameters or size, even when lignin percentage is decreased. It is worth noting that this occurs regardless the species and the position of the enzyme whose gene has been suppressed in the lignin biosynthesis pathway. Indeed, it has been observed when 4CL, the third enzyme of the route, which renders *p*-coumaroyl CoA, is suppressed (Xu et al., 2011); also when CCR, the first committed enzyme of specific lignin pathway; and CAD, which produces coniferyl alcohol, are down-regulated (Tamasloukht et al., 2011; Fornalé et al., 2012).

Despite AtPrx4 has a role in lignification when plants are grown under long-day conditions, this is not so obvious in short-day conditions, as no differences were found between WT and *atprx4* plants, suggesting AtPrx4 is up-regulated in long-day conditions. In fact, a decrease in S units proportion is observed in WT plants grown with short-day conditions, compared to long-day plants. This decrease in S units proportion involves the down-regulation of F5H, COMT and syringyl peroxidases. *F5H*, which is necessary to divert the carbon flux to S monomers, has been shown to be up-regulated along the development when plants are grown under long-day conditions (Ruegger et al., 1999). On the other hand, barely *F5H* expression is noted when plants are grown in darkness, and a sharp increase of its gene expression is found when these plants are changed from darkness to light conditions. This means that S monomers are only formed in specific light conditions, and darkness inhibits the synthesis of S units (Ruegger et al., 1999). The down-regulation of *F5H* in darkness agrees with the unaltered lignification pattern in *atprx4* grown under short-day conditions. Peroxidase regulation by light is unclear, as they can be either up- or down-regulated. However, ZePrx, which AtPrx4 shows most similarity to, has decreased enzymatic activity with sinapyl alcohol when plants are grown in darkness (J Gutiérrez, pers. comm.).

When light conditions are not optimal, plants use the available carbon to synthesize sugars as energy source instead of reallocating carbon into the lignin metabolism, mainly due to the impossibility of reusing the carbon from the lignin polymer. AtPrx4 plays an important role in the overall lignification when light conditions are optimal, but when light conditions are restrictive, xylem lignification (mainly composed of G units) prevails over interfascicular fibre lignification (enriched in S units) and AtPrx4 activity is no longer fundamental. This change in monomer composition, gaining G units, probably involves a loss of stem flexibility, but the maintaining of a functional xylem is saved (Bonawitz and Chapple, 2010).

On the other hand, we have proved that the suppression of just one peroxidase leads to the down-regulation of the whole lignin biosynthesis route, as well as a down-regulation of genes and transcription factors involved in secondary wall thickening (Fig. 5). Moreover, the decrease in lignin content caused a reallocation of carbon fixed in photosynthesis, as deduced from the up-regulation of sinapoylglucose malate sinapoyltransferase (*SMT*), which participates in the sinapate ester biosynthetic pathway from sinapyl aldehyde. The suppression of *AtPrx4* may lead to an accumulation of S monomers precursors, such as sinapyl aldehyde, which might be used for the synthesis of sinapoylmalate. It has been shown that silencing genes in the monolignol biosynthetic pathway substantially affects the flux of intermediates synthesized *via*

interconnected pathways and leads to carbon reallocation (Besseau et al., 2007). Moreover, Herrero et al. (2013b) showed that photosynthetic processes remained unaltered in *AtPrx72* knock-out mutants, in which lignin content was decreased, suggesting that the fixed carbon was used in other routes instead of lignin biosynthesis.

Conclusion

All these results suggest an involvement of AtPrx4 in lignification of cell walls, especially in syringyl monomers formation. The disruption of *AtPrx4* causes a decrease in S units, restricted to interfascicular fibres but, importantly, only when light conditions are optimal. Moreover, the effect of *AtPrx4* disruption is age-dependent, and it is only significant when the elongation process has ceased and lignification becomes active. AtPrx4 emerges as a basic peroxidase regulated by day length with an important role in lignification.

Authors' contributions

FFP carried out the experiments, participated in the study design and helped draft the manuscript. TV participated in short-day experiments. FP coordinated the lignin experiments. MAP participated in the design and coordination of the study, and helped draft the manuscript. ENU participated in lignin experiments, in the design and coordination of the study, and drafted the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2014.11.006.

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